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14. ABSTRACT

In this final report we present our efforts on two or three fronts. We were working on the expression, purification and crystallization of complexes of YopB and YopD with other proteins, like their chaperones. We have made progress in purifying YopB:YopD:SycD complex. However, this is a challenging process and the amount of homologous protein is a problem. Since the expression levels of different components of the complex are different, the stoichiometric ratio is a problem. We will continue to work on this project to successfully end it even though the project period has ended. If successful, this will help in understanding the translocation mechanism of effector proteins.

15. SUBJECT TERMS
Yersinia pestis, Yop complexes, macromolecular crystallography, 3D structure

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Final Report

Introduction

This project was on no-cost extension and here we are presenting the final report.

The overall goal of this project is to determine the three-dimensional structures of proteins and virulence factors from *Yersinia pestis* to understand the mechanism of translocation of effector proteins. *Yersinia pestis* is responsible for the plague and is a potential and emerging biowarfare threat. This organism has evolved a powerful method of delivering effectors into cells of the host immune system. The effectors act to completely circumvent the immune system. Understanding the molecular mechanism of this system is important to get an insight into the process of phagocytosis and inflammation. The effector proteins are secreted into the cells by a type III secretion system which involves a number of proteins. Some of them are actively involved in the delivery system and others are required for pore formation. Information derived from this study will help in developing vaccines and therapeutics for the plague. It will also help in identifying genetically engineered threats. The goal was to study the three-dimensional structures of Yop proteins in complex with their corresponding chaperones. This would allow us study the pore formation and conformational changes the proteins undergo in order to form a pore. By studying the three-dimensional structures of effector proteins we can get an insight into the mechanism of toxification of the cells which would eventually lead to design of inhibitors to block the toxicity. However, the major problem was in making a complex for crystallization.

Body

Work on YopH:

YopH is a key translocated effector of *Y. pestis* that exhibits tyrosine phosphatase activity. The 50 kDa YopH protein is organized into two modular domains separated by a linker region. The N terminal domain has phosphopeptide binding activity and functions as a substrate-targeting domain for the C-terminal catalytic domain. The two

separate domains of YopH have been crystallized and their structures solved (1,2). However, the structure of the native protein has not been determined. It is believed that two domains are connected by a flexible loop or region which prevents crystallization of the intact YopH. It has recently been shown that both the domains have substrate binding sites. We wanted to take advantage of this and design a peptide containing two phosphorylated tyrosine far enough both in sequence and in three-dimensional space to bind to each of the domains. Our idea is to crystallize the intact or mutant YopH with a synthetic peptide. In the last year we have been trying to crystallize these complexes but have had no success yet. We believe that determining the structure of the native protein is important, as it will provide clues into potential functional interactions between the two domains. We have expressed large quantities of YopH for this purpose and are working on crystallization of these with peptides. Particularly we are concentrating on the following synthetic peptides:

Cas peptide (342-371): GSQDI**pY**DVPPVRGLLPNQYGQEV**pY**DTPPMA

Cas peptide (258-287): PATDL**pY**QVPPGPGSPAQDI**pY**QVPPSAGTGH

These peptides are based on the natural substrate, Crk-associated substrate, p130 (p130Cas) of epithelial cells. The p130Cas is one of the proteins with many phosphorylated tyrosines that are critical for many functions like cell-cell interaction, metabolism and immune response. The phosphorylated tyrosines (pY) are shown in bold in the sequences above.

However, multiple trials of crystallization did not yield any diffraction quality crystals. Though the project has ended we will continue to work on this challenging experiment.

Work on YopB, YopD and their complexes

Delivery of secreted effectors such as YopH into host cells infected with *Y. pestis* is controlled by a set of secreted "translocator" proteins. Three proteins are required for the translocation process, LcrV, YopB and YopD. A fourth protein, YopK, appears to function as a negative regulator of translocation. The structure of a mutant form of LcrV has recently been determined. However, the structures of YopB, YopD and YopK have

not been determined. YopB (42 kDa) and YopD (33 kDa) contain predicted transmembrane domains and are thought to form a channel in the host cell membrane. YopK (21 kDa) does not have any recognizable features, and its function remains mysterious. Attempts to overexpress recombinant forms of YopB and YopD in *E. coli* have met with no success, due to their hydrophobic characteristics. As an alternative approach, we have engineered *Yersinia* to secrete these proteins into growth media, and we have investigated the possibility that the secreted forms of these proteins could be used for structural determinations. We worked with relatively small volumes of culture (less than 100 ml) and proteins secreted into *Yersinia* growth media were concentrated by filtration and analyzed by native polyacrylamide gel electrophoresis. Although yields of protein were small (e.g. 10 micrograms) this procedure did provide enough protein for initial studies. The results of these experiments showed that all three proteins, YopB, YopD and YopK, were secreted as soluble proteins. However, all three proteins migrated as multimeric forms on the gels. YopB and YopD formed multimeric ladders of bands on the gels, and YopK ran as a broad band at a very high molecular weight. Thus, although the secreted forms of YopB, YopD and YopK are soluble, the multimeric forms of these proteins made protein crystallization and X-ray determination challenging. Preliminary results indicated that YopB and YopD are forming homo- and hetero-oligomers. We plan additional studies to determine if the hetero-oligomeric forms of YopB and YopD are suitable for crystallization studies. However, we are also trying to express YopB and YopD individually as membrane proteins but expression has not been successful so far.

Since it was very difficult to express and purify YopB and YopD alone, we set out to work on complexes of YopB and YopD with their chaperone, in this case SycD. We have cloned YopB and YopD in pET vector. However, expression of these proteins in *E.coli* is still a challenge. The quantity that we obtained was not enough for our crystallization trials. In addition to getting YopB:YopD complex by co expression, we are also trying another technique. SycD is an intrabacterial protein chaperone. It has been shown that SycD binds to YopB. We are now attempting to co-express YopB with SycD. For this we have cloned YopB with and without histag and SycD with and without histag. When they

are expressed together neither of them is toxic to the cell unlike when YopB alone is expressed.

As reported in the last annual report, though we get some expression of the complex, the amount of protein expressed is not enough for crystallization. We are optimizing the conditions expression for better yield. In addition to continuing our work from last year we have undertaken to express YopB-YopD-SycD complex as described below.

Expression of His-SycD-YopB-YopD

A new construct has been made including LcrH/SycD, YopB and YopD ORFs together (say SycDyopBD) in pET28a vector. It has N-terminal His tag attached to SycD (His-SycDyopBD). This construct was expressed in *E. Coli* cells. The cells were grown in two different methods, one with IPTG induction and other with auto-induction (3). Chloramphenicol and Kanamycin were used as antibiotics in both the procedures.

In the auto-induction protocol cells were expressed in MDG medium initially and transferred to ZYP 5052 medium. The cells were grown at 37°C until the OD reached 0.6 and the temperature was reduced to 20°C for further growth overnight. Harvested cells were lysed and purified with Ni affinity and size exclusion chromatography columns.

Purified protein showed three faint bands in SDS-PAGE corresponding to the proteins, SycD, YopB and YopD (Fig. 1). The intensity of the three bands implied that the proteins were not expressed in a stoichiometric ratio. SycD band is stronger than the other two indicating larger population followed by YopD. The band corresponding to YopB is the lightest. Though it is a poor estimate, the ratio of SycD:YopB:YopD may be deduced as approximately 4:1:2 (Fig. 1).

The IPTG induction protocol also showed the same trend but the overall yield of proteins is less than that of the auto-induction method. So the expression was carried out with auto-induction in most of the experiments.

In order to confirm the expression of the protein of interest, Western blotting was done for YopB and YopD separately using the corresponding antibodies. The Western blot showed the presence of YopB and YopD proteins matching to the bands marked in SDS-PAGE (Figs. 2 & 3).

The size exclusion chromatography has three significant peaks. Peak 1 contained the SycDyopBD complex. The second peak is about half the size of peak 1 and may contain SycD and YopD. Thirdly, a huge peak corresponding to SycD is obtained that is about 100 times stronger than the peak 1. It may be possible that the SycD expression is much more than the complex or the complex formation is poor so that the His-tagged SycD binds to the Ni column more than the complex.

It can be concluded with these experiments that the SycDyopBD complex is expressed but very low in quantity. In fact the crystallization experiments were also made using the complex and SycD samples obtained from size exclusion purification trials. Since the protein concentration is considerably low the crystallization experiments have not been successful, so far.

Improving the yield of proteins especially the SycDyopBD complex has been our focus though unsuccessfully. As before this experiment is till continuing even though the project has ended.

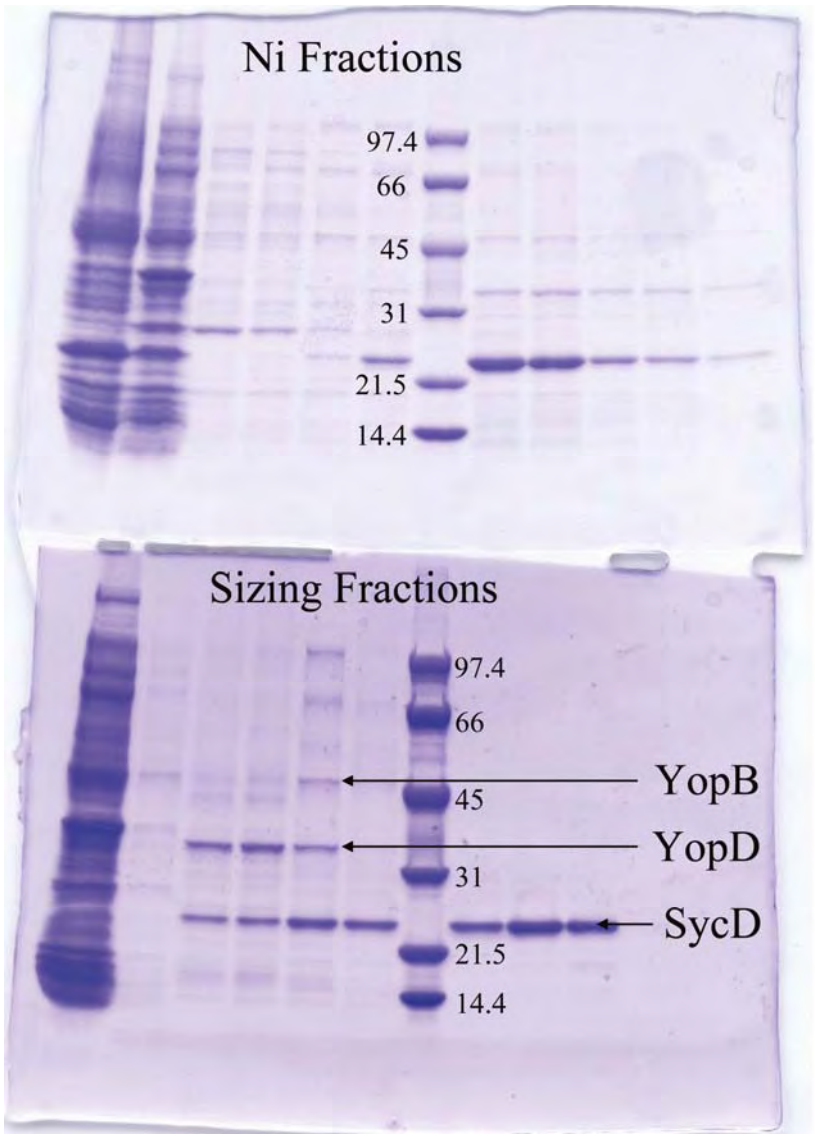

Fig. 1. SDS PAGE gel with the Molecular weight marker. YopB, YopD and SycD are shown with arrow marks.

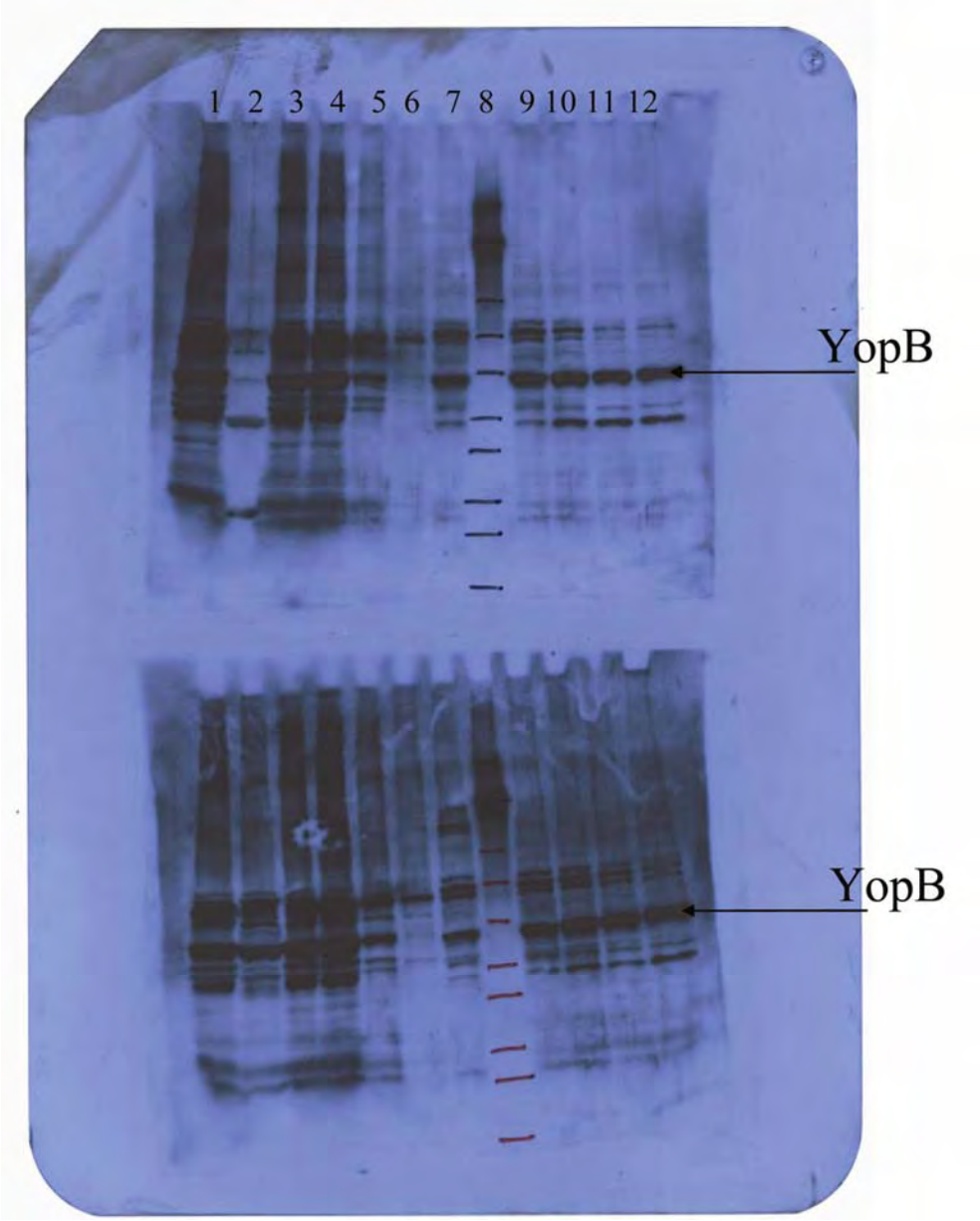

Fig. 2. Western blot showing YopB

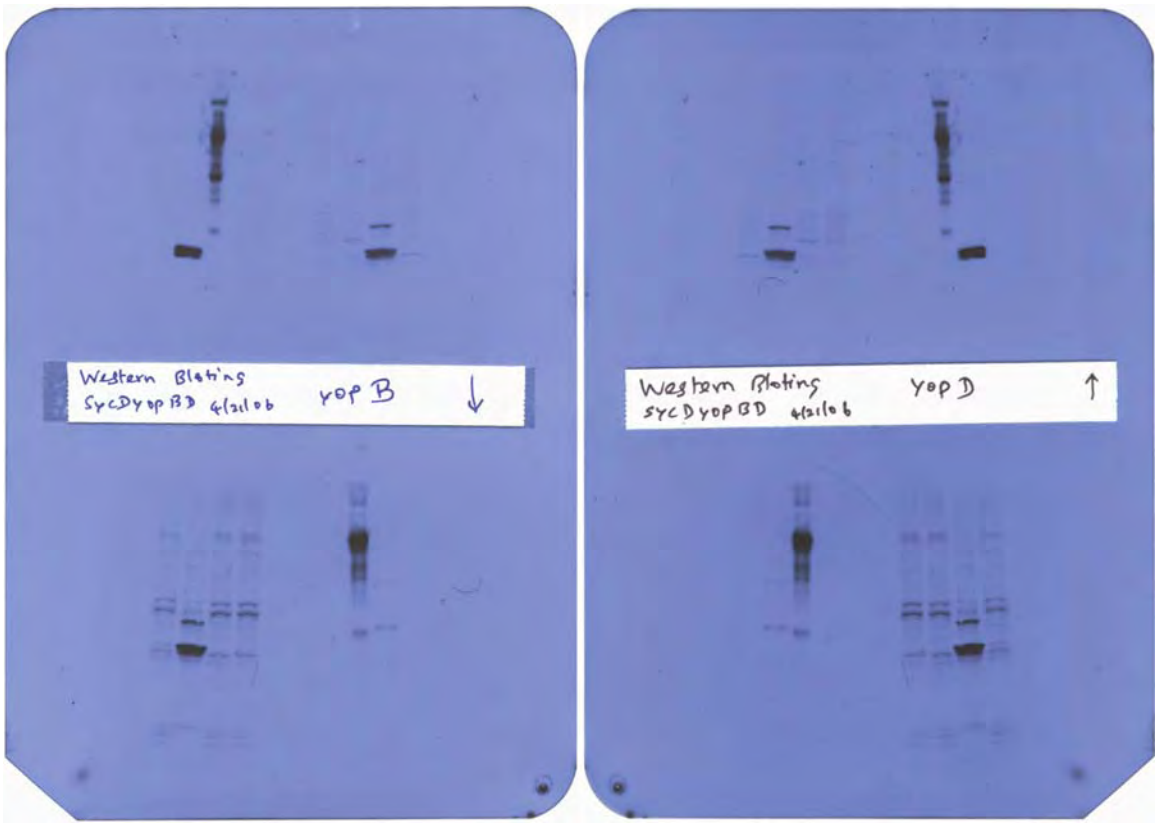

Fig. 3. Western blopt showing the presence of YopD.

Work on YopB-YopD complex:

Preliminary studies indicate that YopB and YopD are secreted into growth media by *Yersinia* in a soluble form. These proteins appear to be forming homo- and hetero-oligomeric complexes. It is possible that the hydrophobic regions of these proteins are buried in the oligomers, which allows them to be soluble in aqueous solutions. Although the homo-oligomeric forms of these proteins may not be suitable for crystallization, we anticipate that the hetero-oligomeric complexes may represent distinct species that could be isolated in pure form and used for crystallization studies. To investigate this possibility, we will purify a secreted form of YopB that contains an N-terminal 6X his tag. We have confirmed that this tagged form of YopB retains full biological activity. The purified YopB will be run on native gels to determine the percentage of the protein

that is in a homo- or hetero-oligomeric form. If we are able to identify a specific YopB-YopD complex on native gels, we will then attempt to obtain sufficient amounts of this complex for structural studies. So far not much success has been achieved towards this. However, this work is being continued with some modifications.

Crystallization of SycD:

SycD is the intrabacterial chaperone of YopD that directs the translocation of the secreted Yop effector proteins across the target membrane. Crystallization of this protein, SycD is also in progress. Expression and purification of this protein is successful and the initial crystallization trials are in progress.

Key Research Accomplishments: We are able to express SycD-YopB-YopD complex. However, the yield is low and we are currently optimizing the expression condition.

Reportable outcomes

None.

Conclusions

We have achieved partial success in purifying Yop B, D and SycD complex but the amount is not enough or homologous enough for crystallization studies. This project is a challenging one and we have been crossing one hurdle after another. We hope to be successful soon. Though the project has ended it is too important to give up at this stage.

Personnel in the Project

1. S. Swaminathan (PI)	Scientist	20% effort
2. S. Eswaramoorthy	Associate Scientist	30% effort

Sub-contract to State University of New York at Stony Brook

1. J. Bliska	Professor	10% effort
2. M. Ivanov	Technician	100% effort

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